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DIFFERENT PATTERNS OF INOSITOL POLYPHOSPHATE PRODUCTION ARE SEEN IN B LYMPHOCYTES AFTER CROSS-LINKING OF sIg BY ANTI-Ig ANTIBODY OR BY A MULTIVALENT ANTI-Ig ANTIBODY DEXTRAN CONJUGATE[1]

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Anti-δ antibody conjugated to 2×10^6 m.w. dextran (dex) stimulates B lymphocyte proliferation at 10,000-fold lower concentrations than that required by the unconjugated antibody. Dex conjugated antibody also stimulates a greater and more sustained increase in intracellular ionized calcium ($[Ca^{2+}]_i$) than does the unconjugated anti-Ig antibody. Inasmuch as inositol phosphate metabolites have been linked to rises in $[Ca^{2+}]_i$, we analyzed by FPLC the relative amounts of the inositol polyphosphates (IP) in these cells. Anti-Ig-dextran induced a threefold greater increase in total IP than did the unconjugated anti-Ig. Furthermore, in cells stimulated by unconjugated anti-Ig there was a transient induction of $I(1,4,5)P_3$ followed by a rapid accumulation of the $I(1,3,4)P_3$ isomer with little accumulation of $I(1,4)P_2$, whereas in anti-Ig-dex-stimulated cells there was prolonged elevation of $I(1,4,5)P_3$ with more accumulation of $I(1,4)P_2$. In addition, levels of $I(1,3,4,5)P_4$ were maintained over a longer period of time in B cells stimulated by anti-Ig-dex than in those stimulated by unconjugated anti-Ig. The enhanced ratio of $I(1,4,5)P_3/I(1,3,4)P_3$ was also seen when suboptimal concentrations of anti-Ig-dex were used which stimulated a level of total inositol phosphate that was similar to that stimulated by the unconjugated anti-Ig. The possibility that the greater stimulation of increased $[Ca^{2+}]$ by anti-Ig-dex than by unconjugated anti-Ig was a predominant factor in influencing the metabolic pathway of $I(1,4,5)P_3$ was excluded. These results show that 1) stimulation of increases in the various IP isomers occurs in anti-Ig stimulated normal B cells as has been shown in B cell lines and 2) that signal transduction and consequent PIP_2 hydrolysis that is stimulated by Ag-mediated cross-linking of sIg is strongly influenced by the extent and type of cross-linking that is induced.

The interaction of a specific ligand with its receptor activates phospholipase C in a number of cell types (1). Phospholipase C hydrolyzes PIP_2[2] into two intracellular second messengers, diacylglycerol and inositol 1,4,5-trisphosphate ($I(1,4,5)P_3$). These respectively activate protein kinase C (2) and mobilize intracellular ionized calcium (3). $I(1,4,5)P_3$ can be further phosphorylated to inositol 1,3,4,5-tetrakisphosphate ($I(1,3,4,5)P_4$) (4–7), which as with $I(1,4,5)P_3$ has also been demonstrated to have an indirect role in regulating the levels of $[Ca^{2+}]_i$ by stimulating Ca^{2+} uptake into the IP_3 sensitive Ca^{2+} pool (5–11). This sequence of events has also been described in normal B cells (12–14) and in B lymphocyte tumors (16, 17) that are stimulated either by Ag or by anti-Ig antibody. The model of anti-Ig antibody-induced B cell activation has thus been viewed as one which closely mimics antigen mediated sIg cross-linking and which provides the advantage of allowing the polyclonal stimulation of all surface Ig$^+$ cells, whereas specific Ag is capable of stimulating only the rare B cells which have specificity for that Ag.

Inasmuch as stimulation of B cells by anti-Ig antibody requires anti-Ig concentrations that are in vast molar excess of sIg molecules, it has not provided the complete model for the induction of Ag-mediated B cell activation because under physiologic in vivo conditions Ag are often present in much more limiting concentrations. To extend the anti-Ig model of B cell activation, we have covalently coupled anti-Ig to a high m.w. dextran to produce a soluble multivalent form of anti-Ig on a carbohydrate backbone (18). This conjugate was found to induce B cell proliferation which is 10 times more than can be obtained by optimal concentrations of the unconjugated anti-Ig. In addition, the conjugate induces B lymphocyte proliferation at a concentration that is 10,000-fold below that which is required for the unconjugated anti-Ig. These results suggest that whereas cross-linking of sIg by either of these two agonists can induce B cells to synthesize DNA, the extent and pattern of cross-linking of surface IgR strongly influences the degree of stimulation (18).

The current experiments were undertaken to determine whether the differences observed in the mitogenicity mediated by these two agonists were also reflected in different patterns, kinetics and magnitudes of inositol polyphosphate production. Although both dextran conjugated and unconjugated anti-Ig antibodies induced

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[2] Abbreviations used in this paper: PIP_2, phosphatidyl-4,5-bisphosphate; $[Ca^{2+}]_i$, intracellular ionized calcium; IP_n (n is the number of phosphate groups), inositol (poly)phosphate

rapid increases in levels of I(1,4,5)P_3, dextran conjugated anti-Ig induced much greater levels of this isomer, which were sustained for significantly longer periods of time. Additional differences were found in the amount and in the ratio of the other inositol polyphosphates that were stimulated. This suggests that the distribution of PIP_2 hydrolysis products that are stimulated by cross-linking of sIg is strongly dependent on the extent and type of cross-linking of sIg that is induced.

MATERIALS AND METHODS

Mice. Female DBA/2J mice were obtained from The Jackson Laboratories, Bar Harbor, ME, at between 6 and 8 wk of age. The experiments reported herein were conducted according to the principals set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Animal Resources, National Research Council, DHEW Publ. No. (NIH) 78-23.

Antibodies and other reagents. The monoclonal anti-T cell reagents, anti-Thy-1.2 (clone 30-H12) (19), anti-CD4 (clone GK1.5) (20), anti-CD8 (clone 53-6.7) (19) were grown as ascitic fluid in nude mice. The mouse monoclonal antibody against rat Ig κ, chain Mar 18.5 (21), was produced by cells grown in tissue culture. The monoclonal antibody (Hδ^a/1) (22) with specificity for the heavy chain of IgD(δ) was grown in nude mice. Antibodies were purified as described previously (23). Conjugation of antibody to dextran was done as recently described (18), and the anti-δ dex conjugate that was used in these experiments had 6 molecules of anti-δ antibody per molecule of dextran. Concentrations of dextran conjugated antibodies that are mentioned in the text reflect only the anti-Ig antibody concentration and not that of the entire dextran conjugate. Thus, in all experiments equivalent protein concentrations of anti-δ and anti δ-dex are studied. Ionomycin was obtained from Calbiochem (San Diego, CA); lithium chloride and EDTA were from Sigma Chemical Co. (St. Louis, MO); HEPES buffer 1 M pH 7.4 was from M.A. Bioproducts (Walkersville, MD); sodium sulfate and zinc sulfate were from Fisher (Springfield, NJ).

B cell purification. Resting B cells were prepared as described previously (18). Briefly, spleen cell suspensions from 30 mice were treated with a cocktail of rat antimouse T cell reagents for 30 min on ice. Cells were then washed once and resuspended in media containing a mouse antirat κ (21) mAb together with complement for 45 min at 37°C. Resting B cells were then isolated from the suspensions by centrifugation over Percoll density gradients. Cells were stained with fluorescein conjugated anti-δ and anti-Thy-1.2 antibodies and were found to contain greater than 95% sIgD$^+$ and no detectable Thy-1.2$^+$ cells.

Analysis of inositol phosphates. Analysis of total inositol polyphosphates was carried out as described previously (17). Identification of specific inositol polyphosphates was done as follows: resting B cells were cultured overnight at a density of 5×10^7 cells/ml in 30 μCi of ^{3}H-myo-inositol in RPMI with 10% FCS. Cells were recovered and washed three times in phenol red-free RPMI with 10% FCS in the presence of 10 mM lithium chloride. Cells were resuspended at 2.5×10^6 cells/ml in the same media and aliquoted at 0.2 ml/microcentrifuge tube, and prewarmed to 37°C for 0.5 h. Anti-δ or anti-δ-dextran (anti-δ-dex) was then added for the indicated time. At the end of the stimulation time cells were spun briefly for 5 s in a microcentrifuge. All media was then removed by aspiration, and the cell pellet resuspended in 0.75 ml of chloroform:methanol (1:1), followed by the addition of 0.25 ml of water and then 0.25 ml of chloroform and an additional 0.25 ml of water. Tubes were then vortexed and respun in the microcentrifuge for 2 min. The water layer was transferred to fresh polypropylene tubes and dried at 37°C under nitrogen. Dried samples were resuspended in buffer "A" for FPLC separation.

Separation of inositol polyphosphates was done on a Pharmacia Mono Q FPLC column by a modification of the method of Meek (24). Buffer "A" was 0.1 mM zinc sulfate, 10 mM HEPES, and 0.1 mM EDTA. Buffer "B" was the same as buffer A with the addition of 0.5 M sodium sulfate. Samples were loaded in buffer A and allowed to flow into the column for 4 min at 1 ml/min. From 4 to 50 min there was a linear gradient to 6% buffer B. At 50 min there was a stepwise jump to 14% buffer B. The linear gradient then continued to 75 min to give a concentration of 15% buffer B. At 75 min there was a step jump to 46% buffer B followed by a linear gradient to 80% B over the next 11 min, and to 100% B by 91 min. Buffer B was then run for 15 min at 100% and then decreased in a single step to 0%. The column was reequilibrated with buffer A for 15 min before the addition of the next sample. Fractions of 1 ml were collected for 89 min. To each fraction 3 ml of Ready Value scintillation fluid (Beckman Instruments Inc., Fullerton, CA) was added and radioactivity was determined by liquid scintillation spectroscopy. The column was standardized by elution of standards, inositol 1-phosphate, inositol 1,4-bisphosphate, and inositol 1,4,5-trisphosphate (Amersham Corp., Arlington Heights, IL), inositol 4,5-bisphosphate (a kind gift from Drs. E. Bonvini and C. Brando, FDA, Bethesda, MD), inositol 1,3,4-trisphosphate and inositol 1,3,4,5-tetrakisphosphate (New England Nuclear, Boston, MA). The inositol polyphosphates eluted sequentially from the column: I(1)P_1 after 5 to 14 min, I(4,5)P_2 after 36 to 38 min, I(1,4)P_2 after 46 to 48 min, I(1,3,4)P_3 after 66 to 68 min, I(1,4,5)P_3 after 70 to 72 min, and I(1,3,4,5)P_4 after 80 to 84 min (Fig. 1). Each experiment was repeated three times and representative results are shown.

Analysis of intracellular ionized calcium. Measurements of $[Ca^{2+}]_i$ in single cells were performed using indo-1 as the indicator dye as described in detail previously (25).

RESULTS

Anti-IgD antibody-dextran conjugate (anti-δ-dex) is potent mobilizer of intracellular ionized calcium. Evidence has accumulated that the hydrolysis of PIP_2, with subsequent elevation of intracellular ionized calcium and activation of protein kinase C, is a central mechanism by which specific Ag and anti-Ig induce B cell activation (12–17). Inasmuch as we have recently shown that anti-δ-dex is a substantially more potent inducer of B cell proliferation than is unconjugated anti-δ antibody (18), we wished to evaluate whether this difference was also reflected at an earlier stage in the activational pathway of B cells. To this end we used flow cytometric analysis of indo-1 labeled B cells to measure calcium mobilization induced by these two similar compounds. When cells were stimulated with 100 μg/ml of unconjugated anti-δ antibody, there was a fivefold transient increase in the concentration of calcium from a resting level of 130 nM to approximately 700 nM (Fig. 2). This decreased rapidly to approximately 250 nM after 2 min of stimulation. In contrast, when a 10-fold lower concentration of anti-δ-dex was used to stimulate the cells $[Ca^{2+}]_i$ reached a mean maximum concentration of approximately 800 nM and, at the termination of the experiment (6 min), the average cell had approximately 400 to 500 nM of $[Ca^{2+}]_i$. This difference in the mean response was not due to recruitment of additional responding cells, as more than 90% of cells responded in both conditions (not shown). The early and the late rise in $[Ca^{2+}]_i$ that are stimulated by both of these agonists are inhibited to varying degrees by EGTA. The early peak response that is stimulated by both agonists within 30 s are inhibited by 25%, whereas the later sustained increases induced by these agonists are inhibited by 90% (not shown). This supports the finding of others (10, 13) who demonstrated that the early rise reflects IP_3-mediated mobilization of calcium from intracellular stores, whereas the later rise reflects influx of calcium from extracellular sites.

Analysis of inositol polyphosphates induced by anti-δ and anti-δ-dex. We wished to determine whether differences in the pattern of calcium mobilization of these two different sIg cross-linking agonists would correlate with differences in the amount or in the ratio of I(1,4,5)P_3 and IP_4 that were induced after B cell stimulation by these mitogens since these inositol phosphates have been demonstrated to influence $[Ca^{2+}]_i$ mobilization. ^{3}H-myo-inositol labeled B cells were stimulated in the presence of lithium either with anti-δ or anti-δ-dex for periods of time ranging from 10 s to 5 min. Water soluble inositol

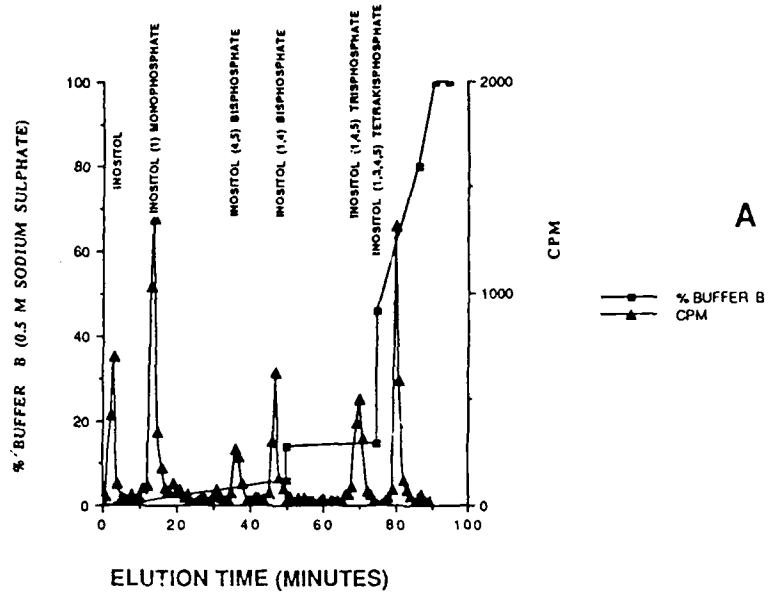
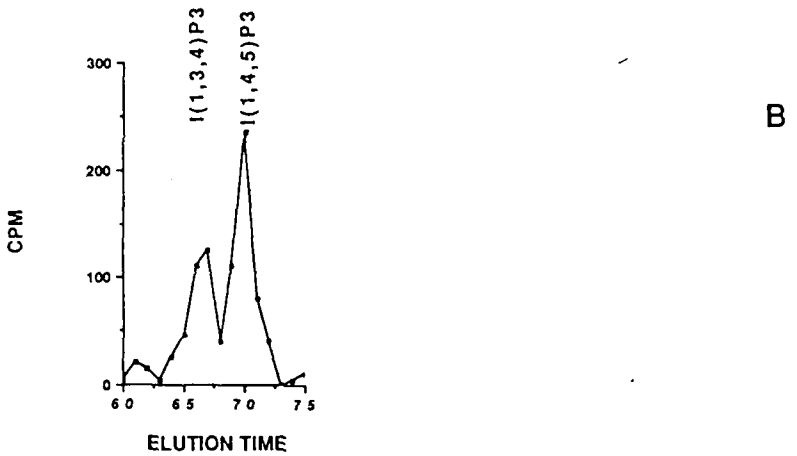


Figure 1. FPLC separation of inositol polyphosphate standards. A mixture of ^{3}H-inositol polyphosphate standards was loaded onto a Pharmacia *mono* Q column. The IP were then sequentially eluted from the column by increasing concentrations of sodium sulfate (buffer B). The results are shown as cpm on the right hand Y axis and time on the X axis. The left hand Y axis shows the change in concentration of buffer B vs time on the X axis.



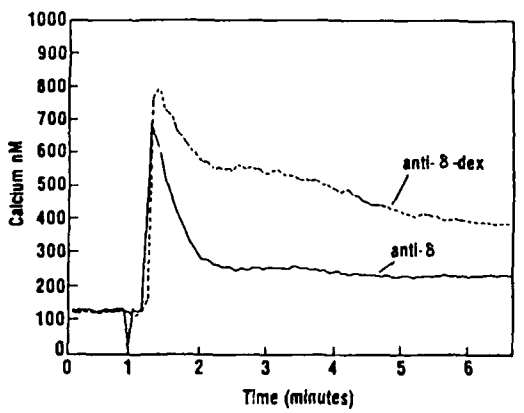


Figure 2. Changes in $[Ca^{2+}]_i$ in B lymphocytes stimulated with anti-δ or anti-δ-dex antibody. Resting B lymphocytes were prepared and loaded with indo-1 as described and then analyzed to determine the ratio of violet:blue fluorescence. At the end of 1 min cells were stimulated with either 100 μg/ml of anti-δ or 1 μg/ml of anti-α-dex and the analysis continued for a further 5 min 40 s. Results are expressed as the mean $[Ca^{2+}]_i$ of the population vs time.

polyphosphates were then extracted and applied to a Pharmacia (Pharmacia Fine Chemicals, Piscataway, NJ) FPLC for detailed analysis. Unconjugated anti-δ was found to induce increases in the levels of IP_1, IP_2, IP_3, and IP_4 (Fig. 3*A*). Although the levels of the IP_1 continued to rise throughout the course of the experiment, as might be expected as a result of the inhibitory effect of lithium on the inositol phosphate monophosphatase (26), the amount of IP_2 remained relatively constant, and that of IP_4 decreased. The kinetics of IP_3 induction (Fig. 4, which are data extrapolated from Fig. 3) was comparable in some regard to that seen in other cell types. Thus, the $I(1,4,5)P_3$ isomer was stimulated to increase within 10 s after stimulation but was rapidly converted to $I(1,3,4)P_3$, so that by 2 min more than 90% of IP_3 was present as the $I(1,3,4)P_3$ isomer.

The pattern of inositol phosphates stimulated by the anti-δ-dex (Fig. 3*B*) was significantly different than that stimulated by unconjugated anti-δ in the following ways: 1) The degree of PIP_2 hydrolysis induced, as judged by the total amount of inositol phosphate, was significantly greater and could be stimulated by lower concentrations of anti-δ-dex. 2) Although there was an increased in IP_1

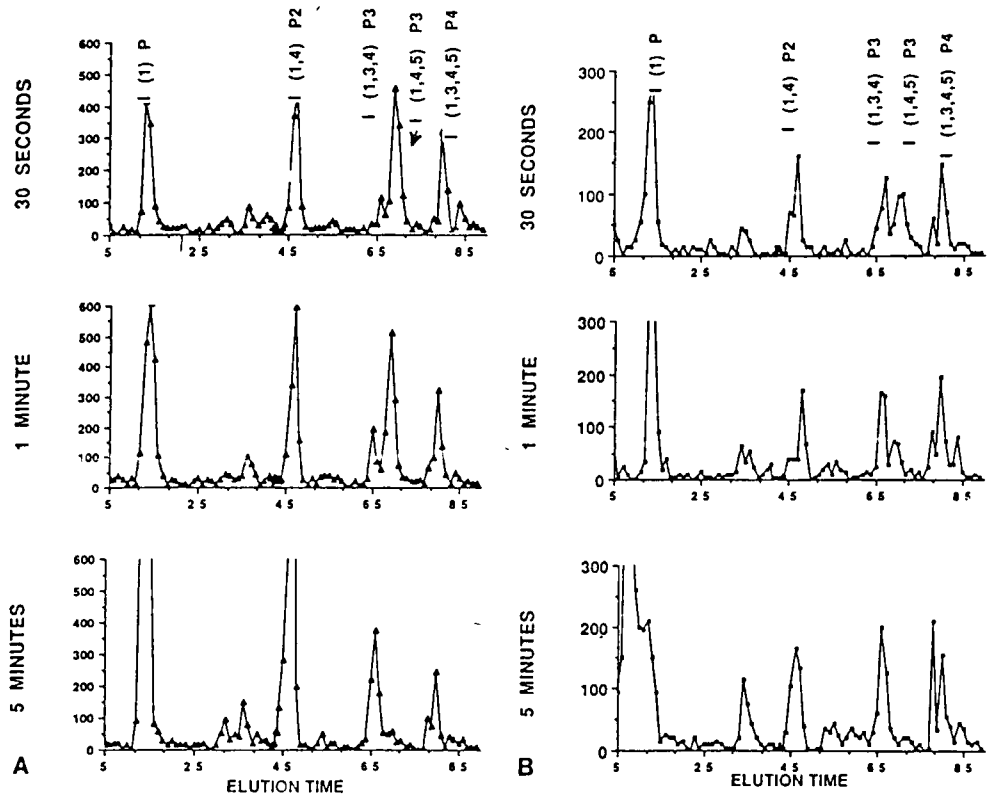


Figure 3. Analysis of inositol polyphosphates induced in B lymphocytes by anti-δ or anti-δ-dex antibody-mediated stimulation. Resting B lymphocytes were prepared and labeled overnight with ^{3}H-myo-inositol. Labeled cells were washed as described and then stimulated with *A*) anti-δ-dex (1 μg/ml) or *B*) anti-δ (10 μg/ml) for the times shown. IP were isolated and analyzed by FPLC. The results are expressed as the cpm of individual 1-min fractions collected during the separation run. Comparable results were observed when anti-δ was used at 100 μg/ml.

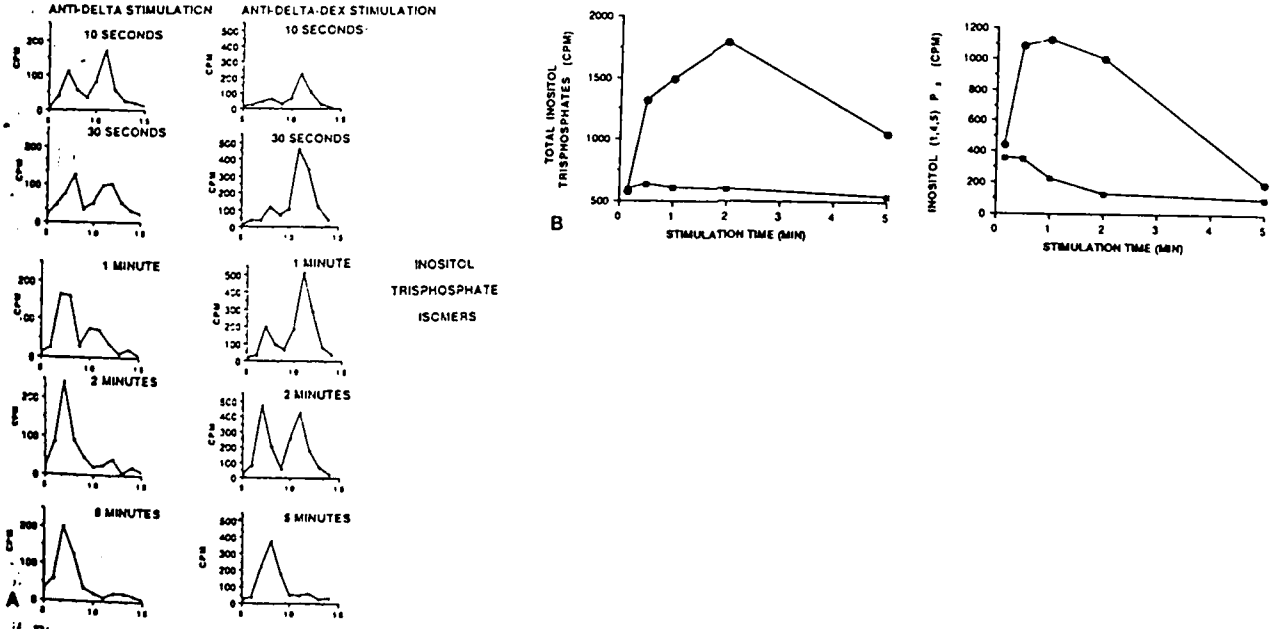


Figure 4. Analysis of inositol trisphosphate isomers induced in B lymphocytes by anti-δ or anti-δ-dex antibody-mediated stimulation. The data from the experiment depicted in Figure 3, together with additional time points, were replotted to compare the patterns of IP_3 isomers that were stimulated under the different experimental conditions (*A*), and the total IP_3 release stimulated over time by anti-δ-dex (●) and anti-δ antibodies (■) (*B*, left panel) is compared to the total amount of I(1,4,5)P_3 (*B*, right panel) that is stimulated by these antibodies.

throughout the course of the experiment as was also noted with unconjugated anti-δ, the amount of IP_2 continued to rise throughout the course of the experiment in cells stimulated by anti-δ-dex but not in those stimulated by unconjugated anti-δ. 3) In anti-δ-dex-stimulated cells there was a gradual increase in levels of I(1,4,5)P_3, which peaked at 1 min after stimulation and stayed elevated for an additional 4 min. There was a preponderance of the I(1,4,5)P_3 isomer to that of the I(1,3,4)P_3 isomer for the first 2 min after stimulation at which time there were approximately equivalent amounts. At this time point, unconjugated anti-δ-stimulated B cells contained no detectable I(1,4,5)P_3 isomer in B cells stimulated with unconjugated anti-δ. These results are in support of our previous observations that anti-δ-dex stimulates greater and more prolonged increases in $[Ca^{2+}]_i$ than does unconjugated anti-Ig. They also suggest that these agonists whereas stimulating comparable pathways of activation, do nonetheless show differences in patterns of inositol phosphate production.

Inasmuch as these experiments were all done in the presence of lithium, which could conceivably have affected these findings, the experiments were repeated in the absence of lithium (Fig. 5). The results demonstrate that lithium had very little effect on the pattern of inositol trisphosphates induced by these two mitogens at these early time points. In all experiments a control unstimulated B cell population was included for analysis of inositol phosphates. Although a small peak of IP_1 was occasionally present (always less than 10% of experimental values) we never were able to detect IP_2, IP_3, or IP_4.

Ionomycin has no effect on inositol polyphosphate induction. There are a number of reports which indicate that the metabolism of the inositol polyphosphates may be affected by the concentration of ionized calcium within the cell (7). Inasmuch as anti-δ-dex antibody induced substantially greater rises in intracellular ionized calcium than did the unconjugated anti-δ, the possibility existed that the differences in the pattern of inositol polyphosphates that were noted may have reflected this difference in $[Ca^{2+}]_i$. To investigate this possibility, we stimulated B cells with unconjugated anti-δ in the presence of the calcium ionophore ionomycin. At this concentration of 1 μm, ionomycin induced an approximately fivefold increase in $[Ca^{2+}]_i$. As shown in Figure 6 there was no difference in the pattern of IP_3 isomers induced in anti-δ-stimulated cells whether in the absence or presence of the calcium ionophore. This indicates that the different pattern of inositol polyphosphates that was stimulated by these two agonists did not result solely from the greater $[Ca^{2+}]_i$ mobilization that was stimulated by anti-δ-dex.

Lower concentrations of anti-δ-dex do not induce substantially different patterns of inositol polyphosphates. We wished to determine whether the differences in patterns of IP_3 that we observed when cells were stimulated with a dextran conjugated or unconjugated anti-δ antibody were solely a consequence of greater hydrolysis of PIP_2 by anti-δ-dex than by unconjugated anti-δ. To this end an experiment was done comparing the inositol trisphosphate isomers stimulated by a wide range of concentrations of anti-δ and anti-δ-dextran antibodies. Cells were stimulated for 2 min with anti-δ or anti-δ-dex antibody at 0.1, 1.0, or 10.0 μg/ml, after which water soluble inositol polyphosphates were isolated and examined by FPLC (Fig. 7). Reducing the concentration

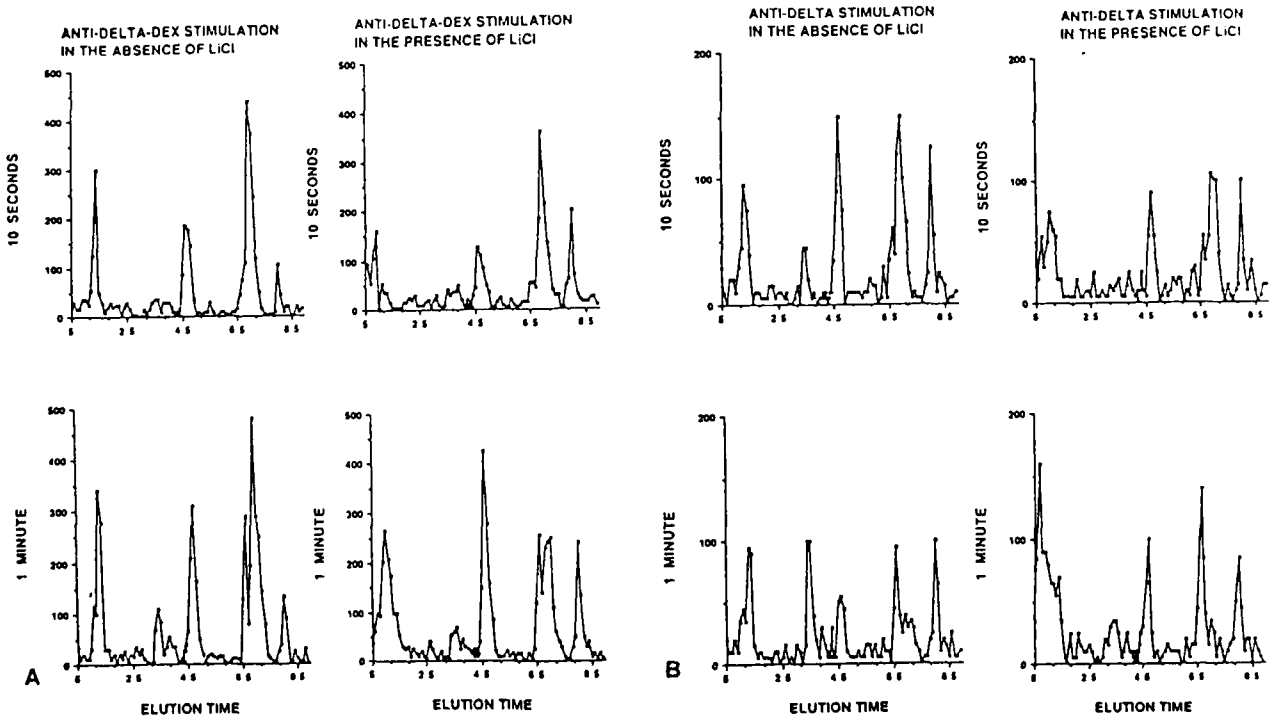


Figure 5. The presence or absence of LiCl does not affect the pattern of inositol polyphosphates produced by stimulation with anti-δ or anti-δ-dex antibodies. Cells were prepared as above and prewarmed in the presence or absence of LiCl (10 mM). Cells were then stimulated with *A*) anti-δ-dex (1 μg/ml) or *B*) anti-δ (10 μg/ml) antibodies for 10 s or 1 min and the IP extracted and analyzed.

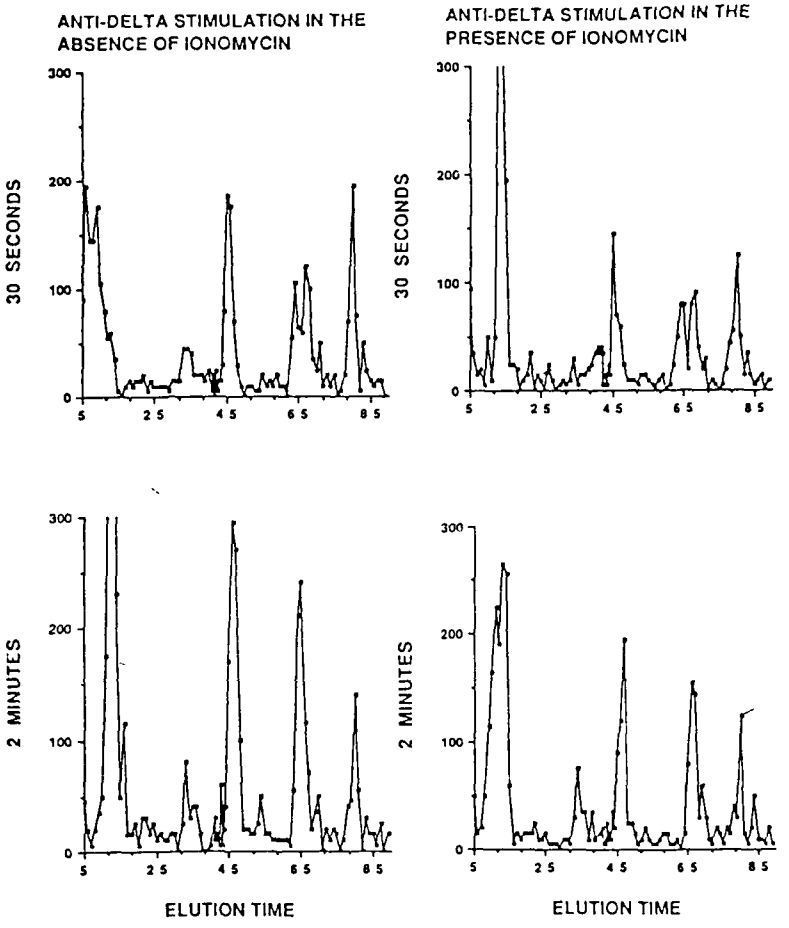


Figure 6. The presence or absence of ionomycin does not affect the pattern of inositol polyphosphates produced after stimulation with anti-δ antibody. Cells were prepared as above and prewarmed. Cells were then stimulated with anti-δ (10 μg/ml) in the presence or in the absence of 1 μM ionomycin for 30 s or 2 min and the IP extracted and analyzed.

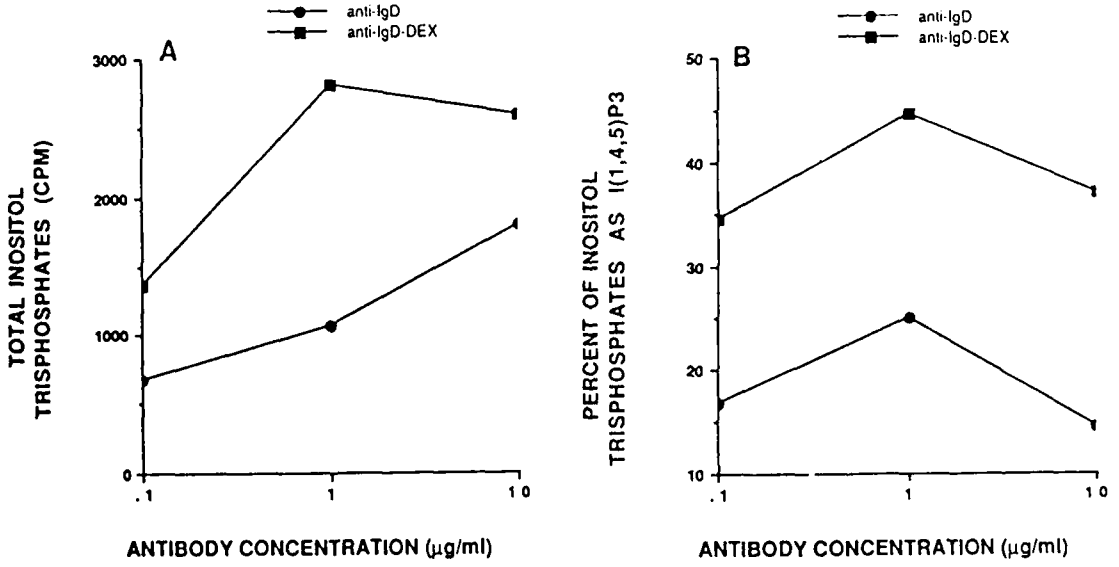


Figure 7. Influence of the concentration of anti-δ-dex antibody on the pattern of the inositol trisphosphate isomers induced in activated B cells. Cells were prepared as above and prewarmed. Cells were then stimulated with either 0.1, 1.0, or 10.0 μg/ml of anti-δ or anti-δ-dex for 2 min and the IP extracted and analyzed by FPLC. The total cpm in the inositol trisphosphate peaks are plotted versus the concentration of the agonist (*A*); the percentage of the inositol trisphosphates as $I(1,4,5)P_3$ isomer was calculated and plotted vs concentration of the agonist (*B*).

of either agonist influenced the quantity of inositol trisphosphates that were stimulated but did not alter the ratio of $I(1,3,4)P_3$:$I(1,4,5)P_3$ (Fig. 7). Thus, 10 μg/ml of anti-δ-dex stimulated 2590 cpm in the trisphosphate fraction of which 37.1% was as the 1,4,5 isomer. Anti-δ-dex at 0.1 μg/ml stimulated approximately one-half as

much total inositol trisphosphate, but did not alter the ratio of $I(1,3,4)P_3$:$I(1,4,5)P_3$. When unconjugated anti-δ was used, only about 20% of the trisphosphates stimulated were present as $I(1,4,5)P_3$ at 2 min. The ratio of $I(1,3,4)P_3$:$I(1,4,5)P_3$ did not appear to be influenced by the amount of the total trisphosphates that were stimulated. Thus, 10 μg/ml of anti-δ stimulated a greater amount of inositol trisphosphates as did 0.1 μg/ml of anti-δ-dex. Nonetheless, the ratio of $I(1,3,4)P_3$:$I(1,4,5)P_3$ did not approach that seen when cells were stimulated with anti-δ-dex.

These data suggest that the relative amounts of $I(1,4,5)P_3$ isomers that are formed after B cell stimulation reflect the mode in which sIg is cross-linked, and this differs significantly when anti-δ antibody constructs with different valencies are used.

DISCUSSION

The avidity of the binding of Ag by anti-Ig antibodies to sIg plays an important, but not exclusive, role in determining their ability to stimulate B cell DNA systhesis (23, 27–32). Whether or not this variable also influences the earlier steps in B cell activation, namely PIP_2 hydrolysis and increases in $[Ca^{2+}]_i$ is less clear. To more carefully examine the activation events that are stimulated within minutes after the cross-linking of B cell sIg by multivalent Ag, we used a dextran conjugated anti-Ig antibody (18) which had specificity for the H chain (δ) of the IgD molecule.

We have previously demonstrated (18) that when anti-δ antibody is conjugated to a high m.w. dextran (m.w. 2×10^6) it stimulates high levels of T cell independent B cell activation at pg/mg concentrations. These concentrations are 10,000-fold lower than those required for the induction of B cell DNA synthesis by unconjugated anti-δ. Inasmuch as these low concentrations more closely mimic the concentrations of the multivalent bacterial polysaccharide T cell independent Ag that are achieved under physiologic in vivo conditions, we believe that this conjugate represents a more valid useful additional model for the studying of T cell independent B cell activation. To determine whether the enhanced B cell mitogenic capability of anti-δ-dex as compared to unconjugated anti-δ is reflected in early activational events we measured increases in $[Ca^{2+}]_i$ that were stimulated by anti-δ and anti-δ-dex. Anti-δ-dex-stimulated cells had significantly greater and more prolonged increases in $[Ca^{2+}]_i$ than did the unconjugated anti-δ stimulated cells (Fig. 2). These differences were observed even when we decreased the concentration of anti-δ-dex by 10-fold (not shown). The later occurring sustained increases in $[Ca^{2+}]_i$ appear to reflect influx from extracellular sites, since it is inhibited by more than 90% in the presence of EGTA.

Inasmuch as it has been shown that anti-Ig stimulates PIP_2 hydrolysis (12–14, 16, 17) it was likely that this early increase in $[Ca^{2+}]_i$ reflected mobilization of calcium from intracellular stores mediated by $I(1,4,5)P_3$. However, it was also possible that the increase in $[Ca^{2+}]_i$ may have reflected an increased influx of calcium which may have been independent of any increases in $I(1,4,5)P_3$ (33). Furthermore, because the only report to date which has analyzed the isomers of inositol polyphosphates that are stimulated after Ag-mediated receptor ligation used a lymphocyte tumor B cell line which was growth inhibited rather than stimulated by anti-Ig antibody (14), we thought it valuable to characterize the production of inositol phosphates in normal resting B cells that become activated by sIg cross-linking. There were significant and consistent differences in the amounts and in the pattern of inositol polyphosphates stimulated by these two agonists. Both stimuli induced rapid increases in IP_1, IP_2, IP_3, and IP_4; however, anti-δ-dex induced greater and more prolonged increases in $I(1,4,5)P_3$ and IP_2 (Figs. 3 and 4) than did anti-δ, as well as a greater ratio of $I(1,4,5)P_3$:$I(1,3,4)P_3$. Although this finding is consistent with the observation that anti-δ-dex induces greater and more prolonged increases in $[Ca^{2+}]_i$ which results from the IP_3-mediated mobilization of calcium from intracellular stores, it does not exclude the possibility that much of the observed increase in $[Ca^{2+}]_i$ may also be contributed to by an increase in calcium influx. It was therefore essential to exclude the possibility that the greater increase in $[Ca^{2+}]_i$ that is stimulated by anti-δ-dex than by unconjugated anti-Ig may itself be a crucial event which influenced the ratio of $I(1,4,5)P_3$:$I(1,3,4)P_3$. The finding that the addition of ionomycin to B cells, at concentrations which increase $[Ca^{2+}]_i$ approximately fivefold did not influence the ratio of $I(1,4,5)P_3$:$I(1,3,4)P_3$ in anti-δ stimulated cells, minimizes this possibility. Another explanation which could account for the enhanced and prolonged $I(1,4,5)P_3$:$I(1,3,4)P_3$ ratio stimulated by anti-δ-dex is that the high levels of $I(1,4,5)P_3$ that are stimulated within seconds after sIg cross-linking may exceed the capability of the 3-kinase to phosphorylate IP_3 to $I(1,3,4,5)P_4$. Alternatively, the higher levels of $I(1,3,4,5)P_4$ may have exceeded the capability of the 5-phosphatase to dephosphorylate IP_4 to $I(1,3,4)P_3$. We have excluded both of these possibilities as being the sole determinants by our observation that lower concentrations of anti-δ-dex which stimulate comparable levels of IP_3 as do high concentrations of anti-δ still stimulate higher ratios of $I(1,4,5)P_3$:$I(1,3,4)P_3$ than does the unconjugated anti-δ.

Why then does cross-linking of sIgD by these two different agonists both of which bind to sIgD and induce capping and modulation, induce differences in patterns of inositol phosphate production? One possibility is that the dextran carrier may be producing a second signal that suppresses hydrolysis of membrane phospholipids. We consider this unlikely because unconjugated dextran does not influence hydrolysis of membrane phospholipids in the presence of unconjugated anti-Ig. Furthermore, although it is possible that differences in the rate of binding within the first 5 min play a major role in influencing hydrolysis of membrane phospholipids by these agonists, we think it unlikely because we have not found significant differences in their binding to resting B cells during this time period (M. Brunswick, and J. J. Mond, unpublished observations). The explanation that we believe is more likely is the manner in which the sIg is cross-linked by these two agonists. The anti-δ-dex complex which has 20 bivalent molecules of anti-δ per molecule of dextran can lead to multipoint binding which does not occur with the unconjugated anti-δ which is only bivalent and thus cannot induce the same degree or stability of crosslinked sIg molecules and may bind different kinetics. Furthermore, upon continued exposure of B cells to anti-Ig antibody, sIg is modulated and re-

moved from the surface of the B cell (23). The residual sIg would be bound more efficiently by the multivalent dextran conjugate than by the bivalent unconjugated anti-δ. Thus, the conjugate may mediate repetitive and more prolonged signaling over a longer period of time. The data presented in our report suggest that this may play a role in influencing hydrolysis of membrane phospholipids. This finding provides an explanation for the enhanced calcium mobilization seen with the anti-δ-dex and may also clarify why a relatively low concentration of anti-δ-dex is a significantly better B cell stimulator than higher concentrations of unconjugated anti-δ. However, the fact that we have observed (34) that even lower concentrations of anti-δ-dex that are mitogenic stimulate neither detectable hydrolysis of membrane phospholipids nor calcium mobilization suggests that other membrane phospholipid independent pathways of activation may also be involved.

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